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Original Article

Positive regulation of placentation by L-amino acid transporter-1 (lat1) in pregnant mice

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Abstract: Placenta plays multi-functions in embryo-uterine dialogue through facilitating gas and nutrient exchange, providing an immunological barrier between the fetus and mother and secreting hormones and growth factors to regulate pregnancy. The successful formation and development of placenta requires invasion and differentiation of trophoblast cells, and any defects would result pregnancy related diseases such as intrauterine growth retardation (IUGR), preeclampsia (PE). Lat1 (L-type amino acids transporter 1) is a major Na^+ independent transporter of large neutral amino acids, including several essential amino acids. It has been showed that amino acid was fundamental regulator on cell function and energy metabolism in early embryonic development. It has been reported that *Lat1* mRNA expressed in zygote, blastocyst during the pre-implantation stages and trophoblast giant cells (TGCs) in post-implantation placenta in mouse. Little is known the role of lat1 on placentation. Our research was to explore the effects of lat1 on the placentation in mouse. The expression of lat1 was detected from day 9 to 18 of pregnancy in placenta. The effects of lat1 on placentation were assessed with inhibitor of leucine transport 2-aminobicyclo-(2, 2, 1)-haptane-2-carboxylic acid (BCH) treatment by uterine horns injection on day 8 (D8) of pregnancy. The protein of lat1 was mainly localized in the cytoplasm of maternal decidual cell, spongiotro-phoblast cell (Sp) and labyrinth (Lab). Inhibition of lat1 transportation activity by uterine horns injection with BCH *in vivo* results in disorder of placental anatomical structure in mid-late pregnancy. These results suggest that lat1 might play an important role in mouse placentation progress.

Keywords: Placenta, L-amino acid transporter-1 (lat1), mice

Introduction

The placenta is the first formed organs during mammalian embryogenesis. It is the site of gas, nutrient exchange and waste products between the mother and the fetus. The placenta also forms immunological barrier to protect fetus from maternal immunological rejection. Besides, it can secrete hormones which can change the physiology of mother and regulate maternal adaptions to pregnancy [1-4]. Although there are some small differences between human and mouse placenta in architecture, it is thought to be quite similar in the molecular mechanism underlying placental development because they belong to discoplacenta. Compared with mouse model, human pregnancy has longer gestation period and been characterized by singleton birth even it is inability to perform experimental treatments and obtain tissue at different gestational ages due to unethical restriction [5]. Because of more advantages in short gestational length and accessibly carrying out experiment, mouse is increasingly used as a model to study the essential elements of placental development. The placenta development of mouse began in the blastocyst on embryonic D4 [6], and then the trophoblast cell which adjacent to the inner mass invade into the endometrium and develop into the ecto-placental cone which develop into functionally and structurally complex placenta with the allantois together [5, 7]. The mature placenta in mice is formed between embryonic

days D10 and D13 when the mature circulation of maternal blood vessels through the placenta has been established [2]. The mouse placenta is consisted of the outer maternal decidua area, a middle spongiotrophoblast layer (Sp), and the inner labyrinth (Lab) three layers [5, 8].

Lat1 (L-type amino acid transporter) is a Na^+ independent amino acid transporter of large neutral amino acid as well as some essential amino acids and high expressed in brain, fetal liver, bone marrow, spleen, testis, ovary and placenta selectivity [9-11]. It has been reported that lat1 might has an intimate relationship with human carcinogenesis. Over expression of lat1 is characteristic of many primary human cancers and may be involved with tumor progression by changing intracellular amino acid or signaling transduction [12-14]. Well-controlled trophoblast invasion at maternal-fetal interface is a critical event for the normal development of placenta which was similar to tumor invasion and shared comparable gene and protein regulator [15]. Lat1 has showed high expression in trophoblast giant cells (TGC's) on D10 and D12 and the highest levels of *Lat1* mRNA appeared in trophoblast giant cells (TGC's) on D8 [16]. Our previous study discovered that lat1 was expressed in mouse uterus during early phase placentation and promoted ectoplacental cones (EPCs) outgrowth *in vitro*, these data suggested that lat1 may participate in the placentation of mouse. In the present study, we explored the expression of lat1 in mouse placenta from D9 to D18; furthermore, we examined the effects of lat1 on placentation *in vivo*.

Materials and methods

Animals and treatments

Adult Kun-Ming female mice, aged 6-8 weeks and weighing 20-25 g, were purchased from Chongqing Medical University and raised in a constant photoperiod (12 h light: 12 h darkness). The mice were fed with standard food and ad libidum water. All animal procedures were approved by the Ethics Committee of Chongqing Medical University. The Guidelines for the Care and Use of Animals in Research were followed. Virgin female mice were mated with fertile males of the same strain to induce pregnancy. The appearance of a vaginal plug on the next day morning was designated day 1 of pregnancy (D1). Pregnant mice were sacrificed for placenta tissues collection from D9 to D18 at 9-10 a.m. respectively. There were at least 10 mice sacrificed in every group.

To observe the effects of lat1 on placentation, mice were randomly divided into five groups (n=3, per group) on day 8 of pregnancy. Experimental groups were injected with (i) inhibitor of lat1 activity, BCH (Sigma, 0.25 μg), (ii) in inhibitor of lat1 activity, BCH (Sigma, 0.125 μg), (iii) NH_4OH (Sigma, 1 mol/L, 5 μl) (iv) saline (5 μl) through uterine horns, (v) blank control group was without any treatment. Mice were sacrificed on D16 at 9-10 a.m. and placenta tissues were collected for the subsequent experiments. There were at least 10 mice sacrificed in every group.

Immunocytochemistry

Placenta tissues from D9 to D18 of pregnancy mouse were fixed in 4% paraformaldehyde for 24 h, dehydrated, and then embedded in paraffin. Sections (5 μm) were cut, dehydrated and rehydrated with graded alcohol/water mixtures. Endogenous peroxidase activity was inhibited by 3% H_2O_2 for 30 minutes at room temperature. Antigen was retrieved by citric acid buffer (PH 6.0, contain 1.8% 0.1 M Citric acid and 8.2% 0.1 M sodium citrate). After washes in PBS, non-specific binding was blocked in 10% normal goat serum for 1 h at room temperature followed by incubation with rabbit anti-lat1 primary antibody (1:500, sc-134994, Santa Cruz Biotechnology) overnight at 4°C for 14-18 h. After three washes in PBS, the sections were incubated with goat anti-rabbit IgG (ZB-2301, Zhongshan Biotechnology, Zhongshan, China) for 60 min at room temperature and then the sections were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (ZB-2301, Zhongshan Biotechnology) for 40 min at room temperature. The secondary antibody was detected with 3, 3'-diaminobenzidine solution (ZLI-9033, Zhongshan Biotechnology). For some sections, primary antibody was replaced with normal rabbit IgG (2 μg/ml IgG instead of primary antibody) to serve as negative controls.

Semiquantitative RT-PCR

Total RNA was isolated from mouse placenta using the TRIzol reagent (Invitrogen) as described in the manufacturer's instruction. Total RNA (2 μg) was reverse transcribed in 20 μl of

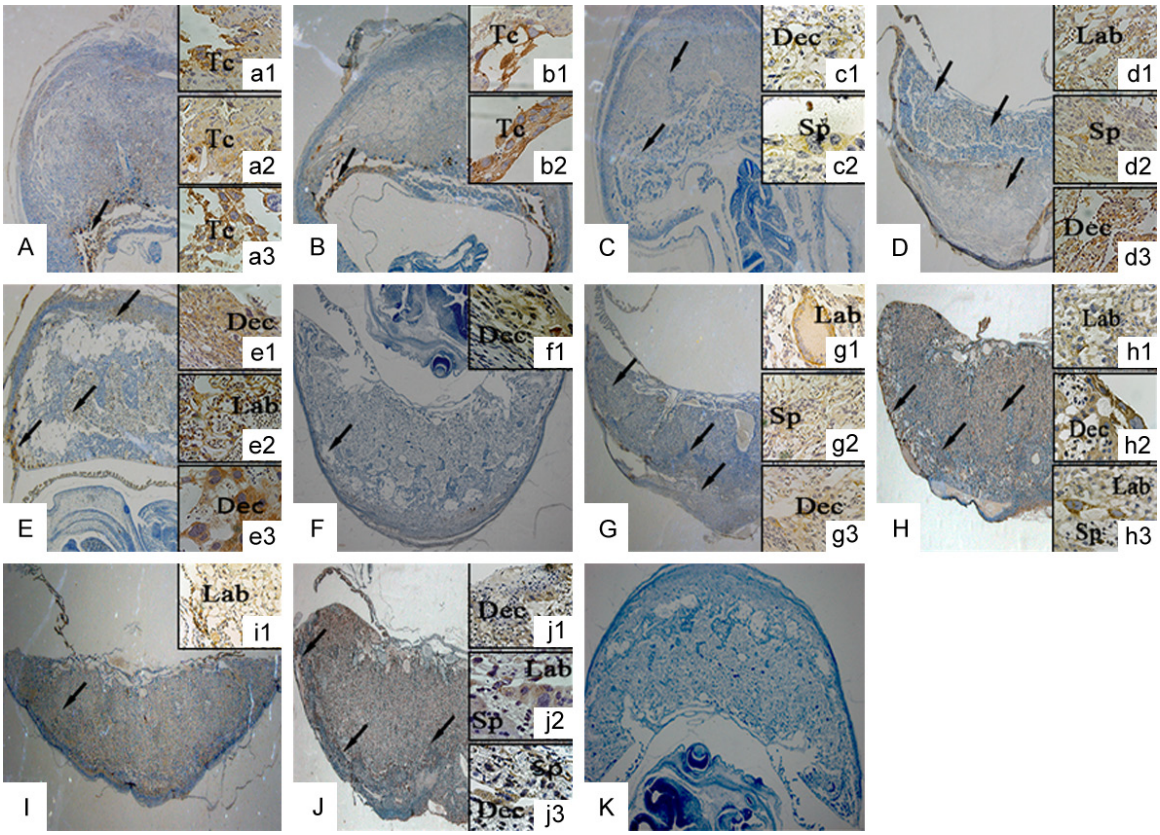

Figure 1. Immunostaining of lat1 in mouse placenta from D9-D18 of pregnancy. (A-J) Immunostaning of lat1 in mouse placenta on D9-D18 of pregnancy, 25 times magnification. (a-j) As shown at their original magnification with 400 times magnification. (K) Negative control which the primary antibody was replaced by IGg. On D9 and D10 the positive expressions of lat1 were detected in the trophoblast cells and decidual cells of maternal-fetal interface. On D11 lat1 mainly expressed in the degraded maternal decidual cells and trophoblast cells of spongiotrophoblast. On D12 and D13, lat1 expressed in degraded decidual cells and the trophoblast cells of spongiotrophoblast and labyrinth three layers. On D14 the lat1 mainly located in the degraded maternal decidual cells. From D15 to D18 lat1 was detected in three cell types of placenta: maternal decidual zone, spongiotrophoblast and labyrinth. But on D17, lat1 mainly expressed in the spongiotrophoblast. Dec Decidual; Sp Spongiotrophoblast; Lab, Labyrinth; Tc Trophoblast cell. Arrow represented the position of the original location magnified in the (a-j) images.

reaction mixture containing 4 μl $MgCl_2$, 25 mM; 2 μl Reverse Transcription 10× Buffer; 2 μl dNTP Mixture, 10 mM; 0.5 μl Recombinant RNasin® Ribonuclease Inhibitor, 15 U AMV Reverse Transcriptase (High Conc.), and 0.5 μg Random Primers (A3500, Promega). The PCR was performed in a total volume of 25 μl containing 12.5 μl GoTaq® Green Master Mix (M7122, Promega), 0.5 μM primers and 1 μl cDNA and was carried out over 22 cycles for *β-Actin* employed as an internal control and 25 cycles for *Lat1*. The thermal cycling conditions were as follows: 94°C for 30 s, 55-59°C for 30 s, and 72°C for 30 s. The primers used in this study include *Lat1* Mus (NM_011404.3) (Forward: 5'-CTTTGTACAGCGGCCTCTTC-3', Reverse: 5'-CAGGACATGACACCCAAGTG-3') and *β-Actin* (Forward primer: 5'-AGCCATGTACGTAGCCATCC-3', Reverse primer: 5'-CTCTCAGCTGTGGTGGTGAA-3').

Western blot analysis

Total protein samples were extracted from mouse placenta with IP cell lysis buffer (Beyotime Biotechnology, Beijing) contained 1% phenylmethylsulfonyl fluoride (PMSF, Beyotime Biotechnology, Beijing) from D9 to D18 of pregnancy. The concentration was obtained with the Bradford assay. Protein samples (30 μg) were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then transferred to polyvinylidene fluoride (PVDF) membrane (Hybond-C, Amersham Bio-sciences, Piscataway, NJ). Membranes were blocked with 5% Albumin Bovine V (BIOSHARP) in tris-buff-

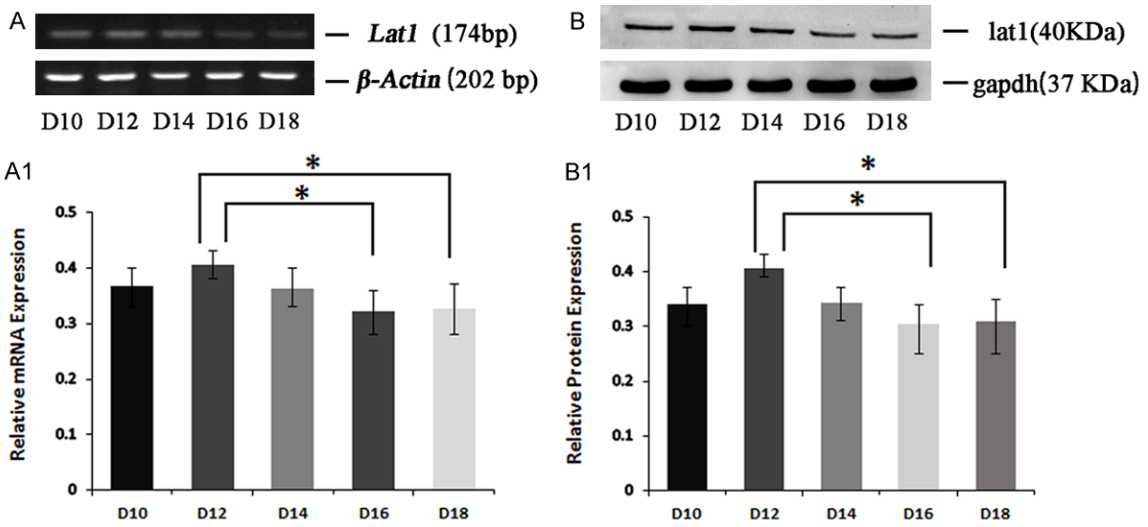

Figure 2. Expression of lat1 in mouse placenta from D9 to D18 of pregnancy. To measure the level of lat1 in mouse placenta from D9 to D18 of pregnancy, D10, D12, D14, D16, D18 placenta tissues were collected respectively. A, A1: Semi-quantitative RT-PCR analysis of the *Lat1 mRNA* in mouse placenta D10, D12, D14, D16, D18 of pregnancy. B, B1: Western blot analysis of the lat1 protein in mouse uterus D10, D12, D14, D16, D18 of pregnancy. On D12 of pregnancy, *Lat1 mRNA* expression level reached maximum, and had statistics difference compared with that on D16 and D18. ($P<0.05$) (the mean ± SE of three independent experiment). *$P<0.05$. The expression tendency of lat1 protein and showed similar with that of mRNA expression.

ered saline-tween 20 (TBST, 0.05% Tween 20) for 1.5 hr at room temperature, and incubated with rabbit anti-lat1 (1:400) and rabbit anti-gapdh (1:1000) primary antibody at 4°C overnight for 14-18 h. After washed with TBST three times incubated with HRP-labeled goat anti-rabbit IgG (1:2000, A0208, Beyotime) for 2 h at room temperature, and then the membranes were washed with TBST for three times and 1× TBS for one time, and then were subjected to enhanced chemiluminescence.

Hematoxylin and eosin (H&E) staining

Mice with BCH treatment in vivo were sacrificed on D16 and placenta tissues were fixed in 4% papraformaldehyde for 24 h, dehydrated, and then embedded in paraffin. Serial sections of 5 μm were cut, dehydrated and rehydrated with graded alcohol/water mixtures and stained with hematoxylin (C0107, C0190, Beyotime Biotechnology) for 30 min and eosin (C0107, C0190, Beyotime Biotechnology) for 5 min respectively and then rehydrated.

Statistical analysis

Each experiment was performed at least three times. One-way analysis of variance followed by a least significant-difference test was used for statistical comparisons among multiple groups. Significant differences were assigned at $P< 0.05$, and highly significant differences were assigned at $P<0.01$.

Results

Expression pattern of lat1 in the mouse placenta

Immunohistochemical staining was shown in **Figure 1**. Lat1 was mainly located in maternal decidual, spongiotrophoblast and labyrinth and continuously expressed in cytoplasm of degraded decidual cells and trophoblast cells located in the placenta. On D9 and D10, lat1 highly expressed in decidual cells and trophoblast cells which were located in the maternal-fetal interface. On D11, lat1 expressed in spongiotrophoblasts, trophoblasts and degraded decidual cells. On D12, D13, D15, D16, and D18, lat1 expressed in the degraded decidual cells and trophoblast cells in three layers of placenta (the outer maternal layer, junctional region, innermost labyrinth) which were fully formed on D12. But on D14, lat1 was mainly expressed in the degraded decidual cells, and on D17 lat1 had the highly expression in the trophoblast cells of spongiotrophoblast.

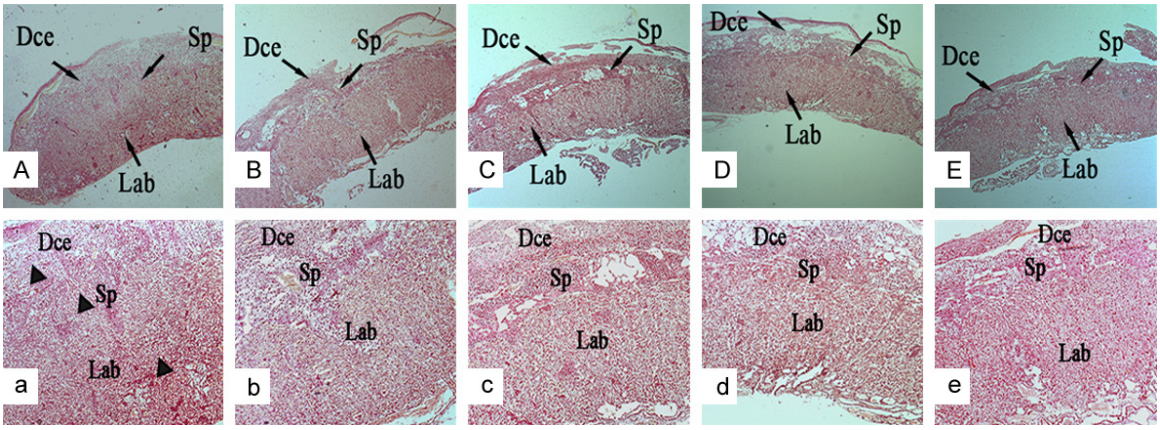

Figure 3. H&E staining showed morphology effects of BCH on D16 placentation after uterine horns injections on D8. A-E: 0.25 μg BCH, 0.125 μg BCH, 1 mol/L NH_4OH, normal saline and normal pregnancy with any treatment, 40 times magnification. a-e: As shown at their original magnification with 200 times magnification. Triangle represented the location of the increased maternal decidual zone and spongiotrophoblast zone; decreased labyrinth thickness; and abnormal differential labyrinth structure. Arrows represent the location of the decidua zone, Spongiotrophoblast and Labyrinth. Dec, Decidual; Sp, Spongiotrophoblast; Lab, Labyrinth.

Expression level of lat1 in the mouse placenta

Expression of *Lat1* mRNA and protein in mouse placenta from D10 to D18 of pregnancy was examined by semi-quantitative RT-PCR and Western blot (**Figure 2A** and **2A1**). The placental total proteins of D10, D12, D14, D16 and D18 of pregnancy were detected by Western Blot. (**Figure 2B** and **2B1**) The peak level of *Lat1* mRNA in placenta was detected on D12, which had statistics difference compared with that on D16 and D18 ($P<0.05$). The similar tendency was present in protein levels.

Morphological effects of BCH on placentation in vivo

To examine the effects of lat1 on placentation, mice were injected with different concentration BCH on day 8 of pregnancy by uterine horns and the placenta was collected on D16. The H&E staining showed that the morphological structure of placenta was disorder, and the placental three layers were not readily discernible (**Figure 3**). Compared with blank control group, placenta with 0.25 μg treatment showed maternal decidual zone and spongiotrophoblast thicker, accompanying with a larger cell density and loses of the sponge sample structure, while the labyrinth became thinner as well as differentiated aberrantly. The 0.125 μg and NH_4OH treatments did not interfere with the placentation process.

Discussion

In the present study, we have investigated the *Lat1* mRNA and protein spatio-temporal expression in mouse placentation. From D11 the spongiotrophoblast has been formed, and the three layers of placenta were fully formed on D12. On D14, the Glycogen cells of spongiotrophoblast began to invade into decidual zone and the chorionic trophoblast began to differentiate into two labyrinth cell types on D15 when the maternal blood spaces expand rapidly, and the labyrinthine volume fraction increased continually until D18 accompanied with decreased junctional zone and decidua basalis [17, 18]. The pattern expression of lat1 in placenta showed coordinately with placenta development. We found that lat1 was mainly located in maternal decidual, spongiotrophoblast and labyrinth and continuously expressed in cytoplasm of degraded decidual cells and trophoblast cells located in the placenta.

Amino acid transport is critical for fetal growth, not only as nutrients but also as regulators for cell motility and function during implantation and placentation [19-22]. Although it was known as twenty amino acid transport systems, the knowledge about them was not understood. System A has been intensively studied in both human and mouse placenta. In human it has been demonstrated that the system A amino acid transporter activity in the microvillus mem-

brane was decreased in gestational diseases complicated with intrauterine growth retardation (IUGR) while increased in gestational type 1 diabetes [23].

Over-expressed of lat1 was characteristic of many primary human cancers and might be related to tumor progression [12-14]. Previous studies have demonstrated the upregulation of lat1 level in prostate cancers, as well as in squamous cell carcinomas, gliomas, urothelial carcinomas, non-small cell lung cancers, breast cancers and pancreatic cancers [24]. Moreover, lat1 expression was one of the most significant predictors of outcome, independent of all other variables, which suggested a key role for lat1 in the biological behavior of tumor [24]. Trophoblast giant cells share the character of highly proliferative and invasive phenotype with cancer [15]. Chrostowski et al. reported that *Lat1* mRNA and protein were detected in all stages of pre-implantation mouse embryos and D8, D10 and D12 three post-implantation development stages. Lat1 was localized to invasive phenotype trophoblast giant cells at D8, which indicated that lat1 might be involved in trophoblast giant cells aggressive phenotype. Lat1 played a key role in mouse trophoblast invasion by mTOR pathway, and active transport of amino acids is required for successful placentation in mouse trophoblast stem *in vitro* [16, 25]. In this study, we found that the level of *Lat1* mRNA and protein in placenta were significantly increased on D12 while decreased on D16 and D18 ($P<0.05$). These results coincided with Chrostowski data and also implied lat1 might assist with the function of trophoblast giant invasive phenotype before placenta maturity fully.

The placenta is composed of many trophoblast cell types: 1) the outer maternal layer, which is composed of uterus decidual cell and maternal vasculature bring s blood to/from implantation site; 2) the junctional region including Sp and TGCs that provide structural support and enable invasion to the uterus; 3) the innermost labyrinth that is the main site of substance exchange and consisted of multinucleated syncytiotrophoblasts that control fetal-maternal transport and sinusoidal trophoblast giant cells that have endocrine functions and act as hematopoietic signaling centers [5, 26]. Placenta mediates concentrative transport of amino acids towards the fetus and placental dysfunction is associated with abnormal amino acid transport [27-30]. In the mouse placenta, substance exchange occurs in the labyrinth, the labyrinth controls the efficient nutrient exchange and transport as well as act as hematopoietic signaling centers [5, 26, 31]. Our previous study showed lat1 positive expressed in EPCs of mouse uterus on D8, and BCH significantly suppressed the EPCs outgrowth *in vitro*. But the relationship between lat1 and mouse placentation *in vivo* was still not stated. Lat1 is an important amino acid transporter for normal physiological activity, so we only chose BCH injection to interfere partly in uterine on D8. Furthermore, BCH was injected in mouse uterine by uterine horns which resulted in the differentiation of placental morphological structure was disturbed and three placental layers were not readily discernible accompanied with enlarged spongiotrophoblast thickness and decreased labyrinth thickness. Thus, we speculate lat1 could regulate the placentation by participating in labyrinth trophoblast cell differentiation.

It has been proved that the labyrinth phenotypes are severe dysfunctional associated with these mutations in many signaling pathways such as Tsc-1, EGFL7, Notch2, Mash2 and so on [32-34]. Amino acid regulation depended on intracellular signaling pathways, such as P13K/Akt/mTOR, Wnt, and STAT3, whether the effects of lat1 on placental formation were collaborated with these signaling factors would need further research.

Conclusion

Overall, our study provided some lines of evidence lat1 were actively involved in placentation progress in vivo. Thus, we could speculate that inhibiting the activity of lat1 can inhibit the placentation progress and lat1 could regulate the placentation by participating in labyrinth trophoblast cell differentiation. But lat1 gene defects can result in gestational diseases such as IUGR and PE directly or indirectly? The role of lat1 in pathophysiological role in human placenta awaits further investigations.

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Disclosure of conflict of interest

None.

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